The melatonin receptor 1A (MTNR1A) gene is associated with recurrent and idiopathic calcium nephrolithiasis

Teresa Esposito, Domenico Rendina, Andrea Aloia, Daniela Formicola, Sara Magliocca, Gianpaolo De Filippo, Riccardo Muscariello, Giuseppe Mossetti, Fernando Gianfrancesco and Pasquale Strazzullo

1Institute of Genetics and Biophysics, Italian National Research Council, Naples, Italy and 2Department of Clinical and Experimental Medicine, Federico II University Medical School, Naples, Italy

Abstract

Background. Experimental evidence indicate that melatonin regulates some renal tubular functions via specific melatonin receptors (MTNRs) located in the kidney of several avian and mammalian species, including humans. We hypothesized that single nucleotide polymorphisms (SNPs) in the melatonin receptor 1A gene (MTNR1A) might influence the risk of calcium nephrolithiasis.

Methods. We performed a systematic analysis of the MTNR1A gene in 246 recurrent calcium stone formers (136 men, 110 women; mean age 40.2 ± 12.0 years; body mass index 25.8 ± 4.5 kg/m²) and 269 healthy controls comparable for age and gender without a history of nephrolithiasis.

Results. Two SNPs in Intron 1 of MTNR1A were significantly associated with calcium nephrolithiasis: rs13140012 (P = 0.0004) and rs6553010 (P = 0.009). The haplotypes resulting from the two SNPs were also differently distributed between stone formers and controls, the haplotype A-T being more represented among stone formers (P = 0.00001) and the haplotype T-C being more common in healthy controls (P = 0.00001). Preliminary functional studies showed that the SNP rs13140012 could modify the binding sites for transcription factors.

Conclusion. The results of this case–control study indicate a strong association between allelic variants of MTNR1A and recurrent calcium nephrolithiasis.

Keywords: association analysis; calcium nephrolithiasis; melatonin; melatonin receptor 1A; single nucleotide polymorphisms

Introduction

Nephrolithiasis is a common disorder which affects ~10% of the adult population in Europe and the United States and is characterized by an elevated rate of recurrence (almost 50% within 5 years from the initial episode) [1, 2]. Kidney stones consist of ~98% crystalline material. Based on the crystalline composition, ~50 types of stones have been recognized; however, calcium oxalate and calcium phosphate salts are by and large the most frequent crystalline components [3, 4]. The large majority of calcium kidney stones are idiopathic [4]. Coe et al. [5, 6] have recently proposed two different pathways for kidney stone formation in idiopathic calcium oxalate and calcium phosphate nephrolithiasis. A first pathway, referred to as overgrowth on interstitial apatite plaques, characterizes idiopathic calcium oxalate nephrolithiasis: as a first step, stone formation requires the expression of Randall’s plaques on renal papillae. Plaques abundance is directly correlated to urinary calcium excretion and inversely correlated to urinary pH and volume [5]. Stone formation and growth are driven by urinary supersaturation of both calcium oxalate and calcium phosphate salts [5, 6]. On the other hand, the proposed pathogenetic pathway for calcium phosphate stone formation is crystal deposit in renal tubules, which occurs more readily the higher the urinary supersaturation of calcium phosphate salts [5]. Therefore, urinary supersaturation, often expressed as the ratio of calcium oxalate and calcium phosphate concentration to its solubility, is the thermodynamic driving force in all kinds of calcium stone formation [4, 7]. All biological processes involved in calcium kidney stone formation depends on the interaction between environmental influences, hormonal factors and genetic susceptibility. Many genes found to be involved in these processes have important systemic functions, namely the genes encoding soluble adenylate cyclase (sAC), vitamin D receptor (VDR), calcium-sensing receptor (CaSR), sodium phosphate co-transporter-2 (NPT-2), chloride channel-5 (CLC-5), transient receptor potential cation channel V (TRPV5) and claudin-16 [8, 9]. Several lines of evidence have led to the hypothesis that melatonin (N-acetyl-5-methoxytryptamine) contributes to regulation of renal tubular function in addition to its postulated role in central nervous system (CNS) physiology. This hypothesis is based on the demonstration of specific, high-affinity melatonin receptors (MTNRs) in the kidneys of several avian and mammalian species.
including humans [10–12]. Immunofluorescent localization studies, carried out in guinea pig, showed that melatonin receptor 1A (MTN1RA) is localized in the renal cortex (predominantly in renal tubules) with relatively minor staining in the glomerulus or medulla [13].

We thus made the hypothesis that some of the biological processes involved in the chemical–physical process of calcium stone formation could be directly and/or indirectly influenced by the actions of the melatonin/MTN1RA biological system. To validate this hypothesis, we analysed the MTN1RA gene in 246 unrelated recurrent calcium stone formers and 269 comparable healthy controls without a history of nephrolithiasis. We analysed calcium stone formers as a whole group considering the common aspects of the pathogenesis and treatment of calcium nephrolithiasis [4].

Materials and methods

Subjects

We studied 246 idiopathic recurrent calcium stone formers [136 men, 110 women; mean age 40.2 ± 12.0 years; body mass index (BMI) 25.8 ± 4.5 kg/m²]. All stone formers reported the elimination or the removal of two or more calcium oxalate (n = 196) or calcium phosphate (n = 50) stones on different occasions during the preceding 4 years. At the same time, a control group of 269 unrelated healthy controls without a history of nephrolithiasis was enrolled (141 men, 128 women; mean age 41.5 ± 11.8 years; BMI 25.3 ± 4.4 kg/m²), including research fellows and employees of Federico II University Medical School, family reference panel (one per family) and patient’s spouses.

Exclusion criteria for both groups were creatinine clearance < 0.75 mL/s/m² or < 0.85 mL/s/m² for women and men, respectively, urinary tract infection, primary or secondary hyperparathyroidism, cystinuria > 0.70 mmol/24 h, gouty diathesis [14], renal tubular acidosis [15], hyperuricemia, chronic diarrhoea states as well as the intake of thiazide diuretics, angiotensin-converting enzyme inhibitors, glucocorticoids or oestrogens. None of the stone formers or controls had rickets during childhood and none had abnormal height or bone deformity as adults.

Both stone formers and controls were born and lived in Campania, a region of southern Italy featuring a high prevalence of nephrolithiasis [16]. All the study participants gave written informed consent before entering the study, which was conducted according to the Declaration of Helsinki. The study protocol received the approval of the local Ethical Committee.

Methods

Stone formers and controls were examined on unrestricted diet at 09:00 h after an overnight fast. At enrolment, the weight and height of each subject were measured, and the BMI was calculated. Two fixed sequence questionnaires aimed at detecting the personal and family history of upper urinary tract stones (the first [17]) and the daily intake of total calories, carbohydrate, fat and proteins on free diet (the second, [18]) were administered to all participants. Fasting urine specimen and 24 h urine collection samples were obtained according to the criteria proposed by Tiselius et al. [19]. Fasting venous blood samples were also taken. Twenty-four-hour urine and blood samples were stored at −20°C and at −80°C, respectively, and analysed contextually to determine the main metabolic risk factors for nephrolithiasis as previously described [20].

Diagnostic criteria

Hypercalciuria was defined as a urinary calcium excretion > 7.5 mmol/24 h in men, > 6 mmol/24 h in women or > 0.1 mmol/kg/24 h [21]; hypocitraturia as a urinary citrate excretion < 1.7 mmol/24 h [21]; hyperoxaluria as urinary oxalate excretion > 444 µmol/24 h [21]; hypocitraturia as a urinary urate excretion < 4.8 mmol/24 h in men and > 4.5 mmol/24 h in women [21]; hypomagnesuria as a urinary magnesium excretion < 2 mmol/24 h [21]; renal phosphate leak as a serum phosphate concentration < 0.80 mmol/L and a maximal tubular renal phosphate reabsorption, normalized for glomerular filtration rate, < 0.70 mmol/L [20].

Kidney stones were analysed using Fourier transform infrared spectroscopy and high-resolution X-ray diffraction [22]. Stones with a calcium oxalate stone content > 70% and a calcium apatite content < 5% were considered calcium oxalate stones [23]. When calcium phosphate salts were the main constituent of stones (> 50%), these were considered calcium phosphate stones [3].

Mutational analysis

Genomic DNA was isolated from whole blood by a standard salting out procedure. Primers sequence and polymerase chain reaction (PCR) conditions are described in Supplementary Table 1. Sequencing procedure was already described [23].

Genotyping

The human MTN1RA gene, located on the chromosomal region 4q35.2, spans a genomic region of 23 913 bases and is split into two exons. Single nucleotide polymorphism (SNP) selection was performed by consulting the HapMap database PhaseII/Rel2, Feb09 on NCBI B36 assembly, dbSNP b126 (http://www.hapmap.org/index.html.en). The default parameters in Haploview were used to create haplotype blocks [95% confidence intervals (95% CI)] as described by Gabriel et al. [24]. Tag SNPs were selected at an r² threshold of 0.80 from all SNPs with minor allele frequency (MAF) > 0.15, allowing us to capture > 80% of the haplotypic diversity of this gene. The variants genotyped in this study were described in Supplementary Table 2.

Expression analysis

To examine the expression pattern of the MTN1RA gene, real-time PCR was performed on a normalized complementary DNA (cDNA) panel (ClinGenome with the LightCycler system DNA Engine Opticon 2 (MJ Research). The QuantiTect SYBR green RT-PCR kit (Qiagen) was used for PCR amplification following manufacturers’ instruction. The expressions were normalized versus γ-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to account for differences in starting material and cDNA reaction efficiency. Each primer pair was specific for the genes of interest. Agarose gel electrophoresis was performed to further confirm the specific PCR products.

MTN1RA transcript primers were forward primer 5’ GACCTCCCGAGACCCACCGA 3’ and reverse primer 5’ GGTGTCGCTACGGTTTCA 3’; the GAPDH primers were forward primer 5’ AGCCATACGGTCAAGACAC 3’ and reverse primer 5’ GATCTCGCTCTGGAAAGATG 3’.

Electrophoretic mobility shift assays

To determine whether the A/T (rs13140012) variant alters nuclear protein–DNA interactions, electrophoretic mobility shift assays (EMSAs) were performed using radiolabelled oligonucleotide probes containing either A or T alleles.

HEK-293 cell nuclear extracts were prepared as described by Granelli-Piperno et al. [25]. The binding reaction mixtures contained 10 ng P32-labelled oligonucleotide probe (105 cpm), 15 µg nuclear extract, 2 µg poly (dI-dC), 100 mM KCl, 10 mM MgCl₂, 20 mM HEPES (pH 7.9), 0.2 mM Ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol, 20% glycerol and protease inhibitor in a total volume of 20 µL. To perform super-shift experiments, 2 µg of the anti-HSF1 antibody (Cellbio) was added to the binding reaction mixture. In competition experiments, a 100-fold molar excess of cold oligonucleotides was added to the binding reaction mixture. Reaction mixtures were incubated for 30 min at room temperature, resolved on non-denaturing 5% polyacrylamide gel, dried and exposed to autoradiography.

The primers used were:

MTN1RA(n131)F-A 5’-GCCCGAGTAGATGAACTCCTGGATAGATTAC-3’. MTN1RA(n131)R-A 5’-TAATGAAAACTACGAAGCTCTCTACATCGCT-3’.

MTN1RA(n65)F-T 5’-GCCGAGGATAGGGGCTGAGATTAGTTAC-3’. MTN1RA(n65)R-T 5’-TAATGAAAACTACGAAGCTCTCTACATCGCT-3’.

Data analysis

Statistical analysis was performed with the SPSS (Statistical Package for the Social Sciences) statistical package version 11.5 and deFinetti program (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl. [26]). Genotype and allele frequencies for the ~386A/G (rs119882), A/G (rs2375801), A/T (rs13140012) and C/T (rs6553010) variants were calculated from
observed genotype counts. The genetic risk magnitudes (effect size) were estimated by calculating odds ratio (OR) with 95% CI.

The analysis of the statistical power was performed using Genetic Power Calculator software (http://pngu.mgh.harvard.edu/~purcell/gpc/cc2.html) [27], assuming a significance level (α) of 0.05, a lifetime risk of 2, and a MAF value for each SNP as calculated in our control group. In the population-based association analysis, the nominal significance threshold was set at $P < 0.05$ and lowered to $P < 0.0125$ after Bonferroni corrections based on the four SNPs analysed. The 95% CI are provided for all OR values. Haplotype frequencies were calculated using the Estimation Haplotype (EH) program (http://linkage.rockefeller.edu/ott/eh.htm).

Clinical and biochemical parameters were expressed as absolute (percentage) values and as mean ± SD for discrete and continuous variables, respectively. Contingency table chi-square tests and analysis of variance with Bonferroni corrections for multiple comparisons were used to test for between-group differences in non-parametric and parametric variables, respectively. All statistical tests were two tailed. Statistical significance was defined as a $P$-value < 0.05.

Results

Human MTNR1A gene expression and polymorphisms selection

To perform the expression profiling of the MTNR1A gene, we analysed different human tissues using a normalized cDNA panel. Significant expression was detected in brain, pancreas, kidney, retina and lymphoblast cells. Low expression levels were detected in heart, liver, placenta and lung cells; no expression was detected in muscle (Figure 1).

To search for a functional variant, we sequenced the coding and the 5′ region of the MTNR1A gene in 20 stone formers and 20 healthy controls. A total of four polymorphic loci were found, including a −386A/G (rs2119882) promoter variant and three coding variants: the non-synonymous change G515A (G166E, rs28383653) and two synonymous changes, A942G (R308R, rs8192550) and A963G (T315T, rs8291551). The numbering scheme for polymorphisms was based on the reported human MTNR1A genomic gene sequence [SNP linked to Gene MTNR1A (gene ID: 4543) via Contig Annotation]. In order to assess the heterozygosis value, the coding variants were genotyped in 50 unrelated subjects. If they exhibited only the major allele, they were removed from further statistical analysis.

Inspecting the HapMap database, we selected three SNPs in different blocks of linkage disequilibrium (LD) along the 22 kb of the MTNR1A gene: rs2375801, rs13140012 and rs6553010. These three SNPs and the −386A/G (rs2119882) promoter variant were typed in a cohort of 246 stone formers and 269 controls samples and analysed in subsequent tests (Figure 2).
Table 1. Allele and genotype frequencies of the MTNR1A SNPs in recurrent calcium stone formers and controls

<table>
<thead>
<tr>
<th>MTNR1A SNPs</th>
<th>Alleles</th>
<th>Sample</th>
<th>Genotypes</th>
<th>Allele 1 vs 2</th>
<th>Genotype 11 vs 22</th>
<th>Genotype 11 vs 12</th>
<th>Armitage’s test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td>12</td>
<td>22</td>
<td>P (OR) (95%CI)</td>
<td>P (OR) (95%CI)</td>
</tr>
<tr>
<td>rs2119882</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>89 (33%)</td>
<td>134 (50%)</td>
<td>46 (17%)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SF</td>
<td>81 (33%)</td>
<td>130 (53%)</td>
<td>35 (14%)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>126 (46.8%)</td>
<td>133 (49.4%)</td>
<td>10 (3.7%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>rs2375801</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>134 (54.5%)</td>
<td>104 (42.3%)</td>
<td>8 (3.2%)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SF</td>
<td>130 (53%)</td>
<td>137 (50.9%)</td>
<td>42 (15.6%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>rs13140012</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>115 (46.7%)</td>
<td>112 (45.5%)</td>
<td>19 (7.8%)</td>
<td>0.009&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SF</td>
<td>90 (33.4%)</td>
<td>137 (50.9%)</td>
<td>42 (15.6%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>rs6553010</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>136 (50%)</td>
<td>111 (41%)</td>
<td>22 (9%)</td>
<td>0.009&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SF</td>
<td>106 (43.1%)</td>
<td>101 (41%)</td>
<td>39 (15.9%)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

<sup>a</sup>SF: recurrent idiopathic calcium stone formers (n = 246); C: controls (n = 269). Statistical significance was defined as a P-value < 0.05; n.s.: P > 0.05. For genotypes, data are expressed as absolute values (percentage). To assess the dosage effect of possessing zero, one or two copies of the risk allele (i.e. an additive effect), the Armitage test for linear trend in proportions was performed on the genotype frequency data. Genetic risk magnitudes (effect size) were estimated by calculating ORs with 95% CI. All statistical tests were two tailed. Statistical power was calculated for all groups using GPC software assuming a significance level (a) of 0.05, a lifetime risk of 2, df of 2 and the allele 2 (minor allele) frequencies of each SNP as calculated in our control group. Statistical power was >0.93 in all cases.

<sup>b</sup>P-value after Bonferroni correction = 0.001.

<sup>c</sup>P-value after Bonferroni correction = 0.002.

<sup>d</sup>P-value after Bonferroni correction = 0.008.

<sup>e</sup>P-value after Bonferroni correction = 0.03.

<sup>f</sup>P-value after Bonferroni correction = 0.01.

<sup>g</sup>P-value after Bonferroni correction = 0.04.

<sup>h</sup>P-value after Bonferroni correction = 0.0008.
Association analysis

We tested the association between the selected MTNR1A gene polymorphisms and recurrent calcium nephrolithiasis. All SNPs analysed were in Hardy–Weinberg equilibrium and showed allele frequencies comparable to those observed in the Caucasian population and reported in the HapMap or SNP browser databases. The statistical power for the four analysed SNPs in the MTNR1A gene was 93% for rs2119882, 97% for rs2375801, 93% for rs13140012 and 97% for rs6553010.

A significant association was observed between two SNPs (rs13140012 and rs6553010) in the Intron 1 of MTNR1A gene and recurrent calcium nephrolithiasis. The allelic and genotypic frequency distribution of the intronic A/T SNP rs13140012 marker in stone formers and controls showed a significant association with nephrolithiasis (alleles: OR 0.6, 95% CI 0.5–0.8, P = 0.0004; genotypes: OR 0.35, 95% CI 0.1–0.6, P = 0.0006). These differences remained significant when the Bonferroni correction for multiple comparisons was applied (alleles P = 0.001; genotypes P = 0.002) and when only calcium oxalate stone formers were compared to controls (alleles: OR 0.6, 95% CI 0.5–0.8, P = 0.0007; genotypes: OR 0.35, 95% CI 0.1–0.6, P = 0.0007).

The analysis of the C/T SNP rs6553010 also showed significant association in both allelic and genotypic distributions (alleles: OR 1.4, 95% CI 1.1–1.8, P = 0.009; genotypes: OR 2, 95% CI 1.1–3.6, P = 0.004) (P = 0.03 and P = 0.01 after Bonferroni corrections) (Table 1; Figure 3). These differences remained significant also when only calcium oxalate stone formers were compared to controls (alleles: OR 1.4, 95% CI 1.1–1.8, P = 0.003; genotypes: OR 2, 95% CI 1.1–3.6, P = 0.01).

We analysed the distribution of the haplotypes formed by the two associated SNPs, rs13140012 and rs6553010. The haplotype frequencies (calculated using the EH program) were significantly different in stone formers and controls (\(\chi^2 = 16.6, df = 3, P\text{-value }0.0009\)). Haplotype-specific testing showed that the haplotype A-T was more frequent in stone formers than in controls (P = 0.00001). Conversely, the haplotype T-C was more frequent in controls than in stone formers (P = 0.00001) (Table 2). These results were confirmed also in calcium oxalate idiopathic stone formers.

Stone formers were then classified according to different rs13140012 and rs6553010 MTNR1A genotypes. As reported in Table 3, we did not find any significant differences in mean values of metabolic risk factors for nephrolithiasis nor in the distribution of stone typology between the different stone formers groups. The distribution of intermediate phenotypes predisposing to calcium nephrolithiasis and the chemical composition of stones were also similar between stone formers groups (Supplementary Tables 3 and 4, respectively).

Electrophoretic mobility shift assays

We performed bioinformatics prediction for associated variants rs13140012 and rs6553010 to evaluate if the nucleotide changes could affect splicing and/or the binding with putative transcription factors. As predicted by transcription factor study (TRANSFAC) [28], only the rs13140012 A > T associated variant seemed to affect the consensus sequence for putative transcription factor binding sites (Figure 4A). To determine whether the rs13140012 A > T alters nuclear protein–DNA interactions, EMSAs assays were performed using radiolabelled oligonucleotide probes containing either A or T. Two distinct complexes were revealed after incubation of the probes with nuclear extract from unstimulated HEK-293 T cells (Figure 4B), and the T probe showed stronger DNA protein-binding activity relative to the A probe. To further assess the binding specificity and the differences in binding affinity between the A and T alleles, a competition assay was performed with unlabelled A or T oligonucleotide. The results revealed that unlabelled T oligonucleotide, but not A oligonucleotide, with 50-fold molar excess partially blocked the binding of the radiolabelled A or T probes with nuclear protein(s). Moreover, to determine if HSF1 was one of the transcription factors bound to the complexes, antibody anti-HSF1 was used in super-shift assays. Under these experimental conditions, none of the complexes were specific for HSF1 (Figure 4B). These data suggest that the A>T polymorphism could affect the binding affinity of several transcription factor(s) and that the variant T allele has a stronger binding strength compared to the A allele.

Table 2. Frequencies of common MTNR1A haplotypes in 246 recurrent calcium stone formers and 269 age-matched, unrelated healthy controls

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>SF (n = 492)</th>
<th>C (n = 538)</th>
<th>P</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-C</td>
<td>249</td>
<td>51%</td>
<td>209</td>
<td>39%</td>
<td>0.0001</td>
</tr>
<tr>
<td>A-T</td>
<td>88</td>
<td>18%</td>
<td>29</td>
<td>5%</td>
<td>0.00001</td>
</tr>
<tr>
<td>T-C</td>
<td>69</td>
<td>14%</td>
<td>175</td>
<td>33%</td>
<td>0.00001</td>
</tr>
<tr>
<td>T-T</td>
<td>86</td>
<td>17%</td>
<td>125</td>
<td>23%</td>
<td>0.022</td>
</tr>
</tbody>
</table>

*SF, recurrent idiopathic calcium stone formers; C, controls; the number of chromosomes examined was reported in parenthesis.
Discussion

The results of this case–control study indicate a strong association between two allelic variants in the MTNRA gene and the recurrent idiopathic calcium nephrolithiasis in a Caucasian population.

Melatonin is a lipophilic hormone, primarily synthesized and secreted by the pineal gland. A well-established function of melatonin is its involvement in the regulation of circadian rhythm and seasonal responses. Moreover, several research papers from different scientific areas suggest that the melatonin biological system participates also in many other physiological and biochemical functions [29, 30]. Four mechanisms of melatonin’s action in mammalian species have been described so far. In addition to its relevant and direct antioxidant activity, melatonin exerts its physiological actions by interacting with nuclear receptors of the orphan family, with intracellular proteins such as quinone reductase 2, calmodulin, calreticulin and tubulin and with specific membrane receptors MTNR1A and MTNR1B [29, 30]. These latter are two G protein-coupled receptors expressed, both singly and together, in various parts of the CNS and in several peripheral organs [29, 30]. They mediate a plethora of intracellular effects depending on the cellular milieu. These effects comprise changes in intracellular cyclic nucleotides [cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate] and calcium levels, activation of certain protein kinase C subtypes, intracellular localization of steroid hormone receptors and regulation of G protein signalling proteins [29, 30]. Circadian variations in the cellular expression of MTNRs have been described. In particular, MTNR messenger RNA (mRNA) levels vary on a circadian basis, with expression levels affected by light and melatonin concentration in plasma. These data indicate that melatonin down-regulates the cellular expression of its receptor population [30].

Our expression studies confirm that the MTNRA gene is expressed in the kidney and supports the hypothesis that the melatonin biological system could play a role in the renal physiology and in the pathogenesis of nephrolithiasis. In fact, a circadian variation in the urinary excretion of different solutes has been demonstrated in healthy subjects: in particular, the nocturnal excretion rates of sodium, potassium, chloride and urate are only 50% of the rates observed during the light period [31, 32], whereas phosphate reabsorption at night exceeds that during the day [33]. Also, urinary pH and calcium showed a circadian variation in healthy subjects [34]. In stone formers, a loss of the circadian rhythmicity of urinary volume and some solutes excretion has been demonstrated [35–40]. This alteration could play a role in the process of renal calcium stone formation, influencing the urinary calcium salt supersaturation during some periods of the day and/or of the year. This hypothesis could explain the absence of significant differences in 24-h mean levels of urinary risk factor for nephrolithiasis in our study population classified according to different rs13140012 and rs6553010 MTNRA genotypes. Of interest, disturbances of the melatonin rhythm as well as alterations in MTNR expression, which are a reflection of a generalized circadian disruption, have been shown in several multifactorial disorders and could play a role in the pathogenesis of
hypertension, obesity and diabetes, which in turn predispose to nephrolithiasis [41, 42].

On the other hand, in HEK-293 cells, the melatonin–MTNR1A system regulates intracellular levels of cAMP, which in turn regulates binding and internalization of calcium oxalate monohydrate crystals by tubular epithelial cells [12, 43].

Based on our data, we do not know if the identified variants have a functional role or if they are in LD with other functional variants outside the gene. However, bioinformatic predictions showed that the T variant of the MTNR1A gene could affect putative-binding sites for transcription factors, altering the consensus sequence. Moreover, the EMSA assays suggested that A>T polymorphism can affect the binding affinity with transcription factor(s) and the variant T allele has a stronger binding strength compared with the A allele. Using expression data available from a small set of human lymphoblast cell lines, we did not find any evidence that rs13140012 directly influences MTNR1A expression (data not shown). However, similar analysis in kidney tissue would be necessary to draw conclusions about the possible effects of the rs13140012 SNP on MTNR1A expression. Other possible study limitations are the relatively small study sample and the use of only one method in the TRANSFAC, which, however, has provided interesting responses.

**Fig. 4.** (A) TRANSFAC prediction of transcription factor binding sites. The upper sequence contains the A allele, the lower sequence contains the T allele (indicated with arrows). (B) EMSA radiolabelled with either rs13140012 A or rs13140012 T probes and HEK-293 cell nuclear extracts (NEs). Lanes 1 and 6, mobility of the labelled probes without NE; Lanes 2 and 7, mobility of the labelled probes with NE in the absence of competitor. A specific nuclear protein binding can be almost completely abolished by 50-fold unlabelled T, but less with A (Lanes 4 and 5; Lanes 9 and 10). Super-shift assays incubating with anti-HSF1 antibody did not show any super-shifted protein complex (Lanes 3 and 8).
In conclusion, our study results indicate a significant association between the MTNR1A gene and nephrolithiasis. Nephrolithiasis is a multifactorial disorder and, consequently, its genetic aetiology is likely to encompass a variety of ‘modest effect’ susceptibility genes. It is possible that variants in the MTNR1A gene may influence the occurrence of the nephrolithiasis and its severity. The rs13140012 A>T variant may act in combination with other yet to be identified functional variants in the gene to influence the nephrolithiasis risk. To our knowledge, this is the first genetic polymorphism investigation to link the MTNR1A—melatonin biological system to calcium nephrolithiasis. Of course, it will be important to study these relationships in other independent populations before asserting the generalizability of this phenomenon.

Supplementary data
Supplementary Tables 1–4 are available online at http://ndt.oxfordjournals.org.

Conflict of interest statement. None declared.

References

13. Song Y, Chan CW, Brown GM et al. Studies of the renal action of melatonin: evidence that the effects are mediated by 37 kDa receptors of the Mel1a subtype localized primarily to the basolateral membrane of the proximal tubule. *FASEB J* 1997; 11: 93–100
27. Purcell S, Cherry SS, Sham PC. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics* 2003; 19: 149–150


43. Lieske JC, Huang E, Toback FG. Regulation of renal epithelial cell 

Received for publication: 9.10.10; Accepted in revised form: 28.3.11